

# Role of Gadd45a in Wip1-dependent regulation of intestinal tumorigenesis

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Conversion of intestinal stem cells into tumor-initiating cells is an early step in *Apc*<sup>Min</sup>-induced polyposis. Wild-type p53-induced phosphatase 1 (Wip1)-dependent activation of a DNA damage response and p53 has a permanent role in suppression of stem cell conversion, and deletion of *Wip1* lowers the tumor burden in *Apc*<sup>Min</sup> mice. Here we show that *cyclin-dependent kinase inhibitor 2a*, *checkpoint kinase 2*, and *growth arrest and DNA damage gene 45a* (*Gadd45a*) exert critical functions in the tumor-resistant phenotype of *Wip1*-deficient mice. We further identified *Gadd45a* as a haploinsufficient gene in the regulation of *Wip1*-dependent tumor resistance in mice. *Gadd45a* appears to function through its ability to activate the Jnk-dependent signaling pathway that in turn is a necessary mediator of the proapoptotic functions of p53 that respond to activation of the  $\beta$ -catenin signaling pathway. We propose that silencing of *Gadd45a* is sufficient to override p53 activation in the presence of active  $\beta$ -catenin under conditions of an enhanced DNA damage response.

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Conversion of normal cells into tumor-initiating cells is an initial step in the course of formation of any tumor.<sup>1</sup> The majority of cells in an organism are terminally differentiated and thus are not capable of conversion into tumor-initiating cells, which requires cellular proliferation. In many instances, proliferative progenitors are programmed to divide only a few times before full differentiation; acquiring oncogenic mutations at this stage may also have a limited impact on tumorigenesis unless the mutation blocks differentiation. Increasing evidence in turn indicates that certain types of cancers originate from adult stem cells, the only proliferative cells that exist in the animal for a significant period of time that may be sufficient to accumulate oncogenic mutations. Several studies have demonstrated the role of adult stem cells as an origin of cancer, at least in certain tissues such as mouse intestine.<sup>2</sup> Stem cell-specific inactivation of *Apc* (*adenomatous polyposis coli*), the major regulator of tumorigenesis in the intestine, results in full-blown polyposis within a few weeks.<sup>3</sup> In contrast, deletion of *Apc* in proliferative progenitors or more differentiated cell types fails to induce sustainable cancer.<sup>3</sup>

In many instances, activation of oncogenes in normal cells sets off the defense mechanisms to protect them from potential transformation.<sup>4</sup> The major pathway that has evolved to perform this function is controlled by a tumor suppressor p53. Depending on the strength of the signal, which in many cases relies on the type of oncogene, p53

activates apoptosis, transient or permanent cell cycle arrest called senescence.<sup>5</sup> Modulation of p53 levels and activity may therefore be critical to the regulation of cell susceptibility to oncogenic transformation. Regulation of p53 by various means to prevent oncogenic transformation may be especially important when particular oncogenic stimuli do not efficiently induce p53. For example, activation of the  $\beta$ -catenin signaling pathway readily induces p53 *in vitro*, but is not sufficient to activate apoptosis in the mouse intestine.<sup>6</sup> Thus, in the absence of appreciable induction of p53, activation of the  $\beta$ -catenin signaling pathway leads to the conversion of intestinal stem cells (ISCs) into tumor-initiating cells and to the formation of polyps. On the other hand, lowering the threshold of p53 activation may induce efficient elimination of cells that have acquired oncogenic mutations, reducing the tumor burden in *Apc*<sup>Min</sup> mice.<sup>6</sup>

Activation of a DNA damage response is an important early step in suppression of tumorigenesis, including colorectal cancer.<sup>7</sup> A similar response can be experimentally imposed by deletion of the wild-type p53-induced phosphatase 1 (Wip1) in mice, which results in activation of ataxia telangiectasia mutated (ATM) and its numerous downstream targets.<sup>8,9</sup> Wip1 is a protein phosphatase 2C family serine/threonine phosphatase that has been implicated in regulation of several signaling pathways, including p53. The gene encoding Wip1, *PPM1D*, is located on chromosome 17q23 and is amplified in a variety of primary human tumors, negatively regulating

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**Abbreviations:** Wip1, wild-type p53-induced phosphatase 1; ATM, ataxia telangiectasia mutated; APC, adenomatous polyposis coli; gadd45a, growth arrest and DNA damage gene 45a; Cdkn2a, cyclin-dependent kinase inhibitor 2a; Chk2, checkpoint kinase 2; Lgr5, leucine-rich repeat-containing G-protein coupled receptor 5; IR, ionizing irradiation; GSK3, Glycogen synthase kinase 3; Mapk, mitogen-activated protein kinase; MKK7, mitogen-activated protein kinase kinase 7; IRES, internal ribosome entry site

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p53.<sup>10,11</sup> *Wip1* deletion leads to tumor suppression in several mouse models of cancer, including mammary gland tumors, B-cell lymphomas, intestinal polyposis, and spontaneous tumorigenesis.<sup>6,8,12</sup> In the intestine, *Wip1* expression was preferentially observed in ISCs at the position +4 from the bottom of the crypt. The *Wip1*-deficient ISCs appeared to be highly sensitive to various stresses, including stress generated in the presence of oncogenes. *Wip1* deletion prevented ISC conversion into tumor-initiating cells in a model of *Apc*<sup>Min</sup>-induced intestinal cancer, suppressing tumorigenesis. In this model, *Wip1* deletion lowered the threshold for p53 activation and eliminated ISCs by inducing apoptosis following activation of the  $\beta$ -catenin signaling pathway. The tumor-suppressive phenotype of *Wip1*-deficient mice was fully abrogated by simultaneous deletion of *p53*.<sup>6</sup>

In this work, we wished to identify the regulators of p53 that could suppress ISC conversion into tumor-initiating cells under conditions of an enhanced DNA damage response, such as occurs in the absence of *Wip1*. We determined that *cyclin-dependent kinase inhibitor 2a* (*Cdkn2a*), *checkpoint kinase 2* (*Chk2*), and *Gadd45a* have an important role in the tumor-resistant phenotype of *Wip1*-deficient mice. We further show that *Gadd45a* (growth arrest and DNA damage gene 45a) is a haploinsufficient gene that contributes to tumor resistance in *Wip1*-deficient mice. *Gadd45a* appears to function via its ability to activate the Jnk-dependent signaling pathway, which in turn mediates the proapoptotic functions of p53 following activation of the  $\beta$ -catenin signaling pathway. Thus, we propose that silencing of a single *Gadd45a* allele may be sufficient to override the DNA damage response in the presence of active  $\beta$ -catenin and may be an early event in the course of colorectal tumorigenesis.

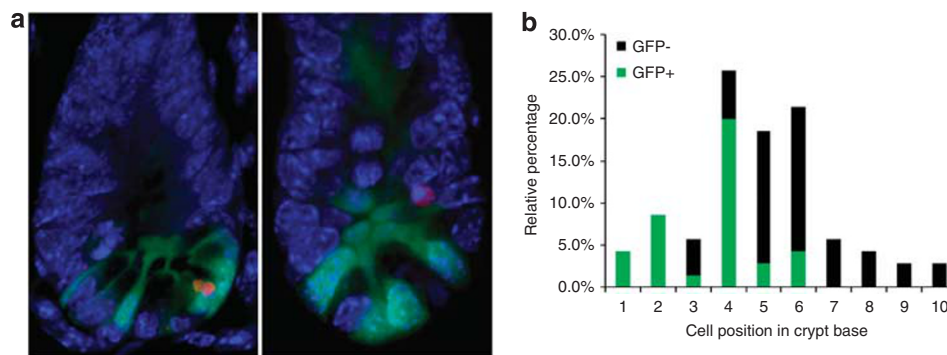
## Results

**Both Lgr5-positive and Lgr5-negative ISCs are sensitive to activation of the  $\beta$ -catenin signaling pathway in *Wip1*-deficient mice.** Emerging lineage tracing data support the existence of two pools of adult stem cells in the mouse intestine.<sup>13,14</sup> While new evidence suggests that the leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5)-positive cells at the base of the crypt provide lineage tracing

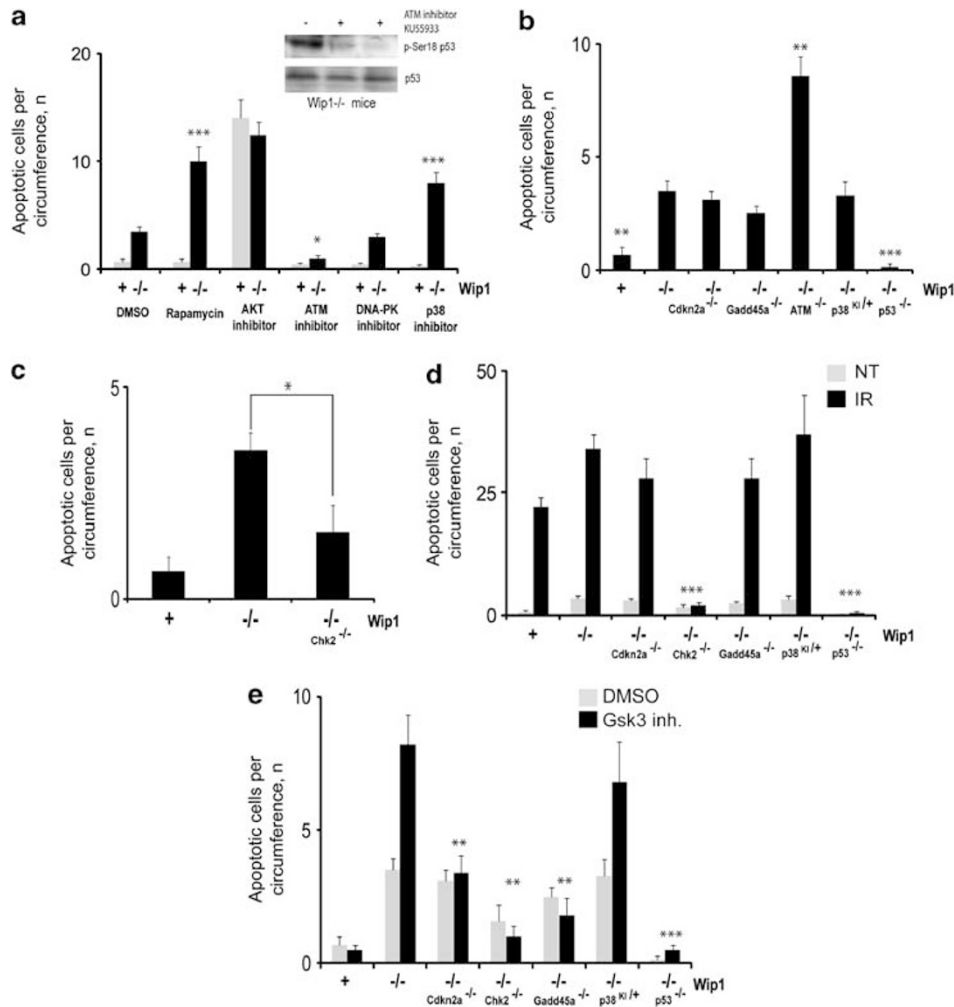
in the intestine and may be ISCs,<sup>13</sup> the long-standing paradigm is that the ISCs are distributed from positions +2 to +6–7 with majority of cells in position +4.<sup>14</sup> Lgr5-positive cells are also commonly found at position +4, supporting the idea of overlap between various stem-cell markers.<sup>13</sup>

We previously found that *Wip1* is preferentially located in +4 cells that express different markers of stem cells such as phospho-phosphatase and tensin homolog, Sox4 as well as retain <sup>3</sup>H thymidine.<sup>13</sup> In turn, *Wip1* deficiency results in enhanced apoptosis of the +4 ISCs in the presence of an active  $\beta$ -catenin signaling pathway, suppressing stem cell conversion into tumor-initiating cells and formation of polyps in an *Apc*<sup>Min</sup> background.<sup>6</sup> To ascertain the relevance of apoptotic cells in *Wip1*-deficient background to expression of another recently identified marker of stem cells, Lgr5, we crossed *Wip1*-deficient mice with mice carrying a cDNA for the enhanced green fluorescent protein (EGFP) knocked into the Lgr5 genomic locus (Lgr5-EGFP-IRES-creERT2 mice<sup>13</sup>). Subsequently, wild-type and *Wip1*-deficient mice, both expressing Lgr5-EGFP, were injected with a specific inhibitor of glycogen synthase kinase 3 (Gsk3) kinase, a kinase that induces phosphorylation-dependent degradation of  $\beta$ -catenin.<sup>6,15</sup> These mice were analyzed for apoptosis 6 h later and we only scored GFP-positive crypts due to mosaic nature of Lgr5-EGFP-IRES-creERT2 mice. Consistent with previously published data,<sup>6</sup> we found a significantly increased apoptosis in *Wip1*-deficient mice after injection of a Gsk3 inhibitor. On average, we found that 43% of apoptotic cells in *Wip1*-deficient mice were Lgr5-positive (Figure 1). Thus, both Lgr5-positive and Lgr5-negative ISCs undergo apoptosis in a *Wip1*-deficient background and therefore are accountable for suppression of polyp formation in the presence of active  $\beta$ -catenin pathway.<sup>6</sup> Our data are also support the conclusion that Lgr5-positive population is more heterogeneous than originally proposed<sup>13</sup> and contains a fraction of cells that are highly sensitive to activation of apoptosis.

**Various signaling pathways control basal and oncogene-induced apoptosis of ISCs in *Wip1*-deficient mice.** Deletion of *Wip1* phosphatase results in enhanced p53-dependent apoptosis of ISCs.<sup>6</sup> We asked whether modulation of various signaling pathways could attenuate the basal



**Figure 1** *Wip1* is required for survival of Lgr5-positive and Lgr5-negative, +4 ISCs. (a) Analysis of apoptosis (red) using TUNEL staining and confocal microscopy was carried out in *Wip1*-deficient mice crossed with Lgr5-EGFP-IRES-creERT2 mice and treated with Gsk3 inhibitor IX. Two representative pictures of double positive (left image) and apoptosis-positive, Lgr5-negative cells (right image) are shown. (b) *Wip1*-deficient mice carrying Lgr5-EGFP were treated with Gsk3 inhibitor IX and apoptosis of Lgr5-positive (EGFP positive, green bars) and Lgr5-negative (EGFP negative, black bars) cells was plotted relative to the position in the crypt base



**Figure 2** Multiple signaling pathways contribute to activation of apoptosis of ISCs in *Wip1*-deficient mice. (a) Analysis of apoptosis in the intestinal crypts based on scoring active caspase 3-positive cells was carried out in *Wip1*-deficient mice treated with different chemical inhibitors as shown in the panel. The inset shows the analysis of phosphorylation of ATM target, p53 at Ser18 (human Ser15) in the presence of ATM inhibitor in intestinal crypts purified from *Wip1*-deficient mice. Two different samples are shown. (b and c) Mice of different genetic backgrounds were analyzed for spontaneous apoptosis in the intestinal crypts. Statistical significance is shown relative to the level of apoptosis in *Wip1*-deficient mice. (d) Apoptosis in the intestinal crypts of mice of different genetic backgrounds was analyzed 6 h after treatment of mice with 6 Gy of IR. Statistical significance is shown relative to the level of IR-induced apoptosis in *Wip1*-deficient mice. (e) Apoptosis in the intestinal crypts of mice of different genetic backgrounds was analyzed 12 h after treatment of mice with Gsk3 inhibitor IX. Statistical significance is shown relative to the level of Gsk3 inhibitor-induced apoptosis in *Wip1*-deficient mice. Significance levels: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

apoptosis of ISCs in *Wip1*-deficient mice. Inactivation of DNA-PK after injecting *Wip1*-deficient mice with previously published effective concentrations of chemical inhibitors had no effect, while inactivation of mTOR, p38 mitogen-activated protein kinase (MAPK) and AKT further enhanced ISC apoptosis (Figure 2a). On the other hand, inactivation of ATM with chemical inhibitor KU55933 lowered the level of phosphorylation of ATM's downstream target, p53 Ser18 (human Ser15; Figure 2a) and was able to reduce the basal apoptosis in *Wip1*-deficient mice (Figure 2a). These results were consistent with the previously described role of *Wip1* as a negative regulator of ATM.<sup>8,16</sup>

To systematically address the role of different signaling pathways in regulation of enhanced apoptosis in *Wip1*-deficient mice, we next analyzed genetically modified mice that had been backcrossed to a *Wip1/Apc<sup>Min</sup>* background.

As we observed that chemical inhibition of ATM reduced basal apoptosis in *Wip1*-deficient intestines (Figure 2a), we crossed *Wip1*-deficient mice to an *ATM*-deficient background. Previous studies have shown that the  $\beta$ -catenin pathway activates p53 through *Cdkn2a*<sup>17</sup>; we therefore generated mice that were deficient for both *Wip1* and *Cdkn2a*. p38Mapk has also been described as a downstream target of *Wip1* that may regulate apoptosis. To downregulate p38MAPK in *Wip1*-deficient mice, we used a knock-in mouse strain expressing the dominant-negative p38<sup>KI/+</sup> allele.<sup>18</sup> We further decided to evaluate *Gadd45a*-deficient mice, as *Gadd45a* participates in regulation of the p38Mapk- and Jnk-dependent signaling pathways.<sup>19–21</sup> In turn, Jnk is an important regulator of p53 and apoptosis under various conditions.

We found that inactivation of *Cdkn2a*, *p38Mapk*, and *Gadd45a* had no apparent effect on basal apoptosis in

*Wip1/Apc<sup>Min</sup>* mice (Figure 2b). On the other hand, deletion of ATM further increased apoptosis, a result in contrast to the outcome of the experiments with an ATM inhibitor (Figure 2a). Although we cannot fully rule out off-target effects of ATM inhibitor, the potential differences may stem from the observation that deletion of ATM, in contrast to a chemical inhibitor, results in profound oxidative stress, which activates p53 and stress kinases including p38 Mapk.<sup>22</sup> This activation may be further enhanced in a *Wip1*-deficient background, as *Wip1* is a negative regulator of both p53 and p38MAPK,<sup>23</sup> creating further toxicity in the intestine and thus enhancing apoptosis of ISCs. As such, we excluded ATM-deficient mice from further analysis due to the complexity of interpreting the results. However, to understand whether a signaling pathway downstream of ATM has a role in intestinal apoptosis in *Wip1*-deficient mice, and considering the limitations of the use of ATM-deficient mice, we turned our attention to *Chk2*, a downstream target and important executor of ATM functions.<sup>24</sup> Deletion of *Chk2* does not exert an effect as deleterious as the enhanced oxidative damage described for ATM-deficient mice.<sup>25</sup> *Wip1*-deficient mice were crossed into a *Chk2*-deficient background; the level of basal apoptosis in these double-mutant mice was reduced and was similar to the level in wild-type mice. This observation supports the idea that both ATM (Figure 2a) and *Chk2* (Figure 2c) are involved in the regulation of basal apoptosis of ISCs in *Wip1*-deficient mice.

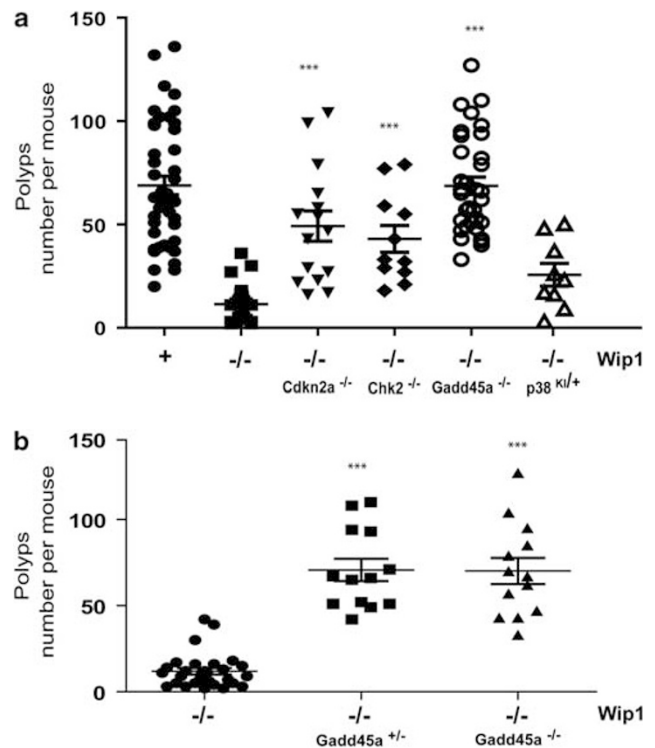
In many instances, *Wip1* deficiency exerts its effects on cell cycle, apoptosis, and ultimately tumorigenesis through activation of ATM.<sup>8,16</sup> ATM, in turn, is an integral part of a DNA damage response induced by ionizing irradiation (IR) and thus, the downstream molecules that attenuate basal apoptosis in *Wip1*-deficient intestines could also participate in IR signaling. To determine whether selected signaling pathways participate in regulation of IR-induced apoptosis in *Wip1*-deficient mice, we irradiated mice of various genetic backgrounds (as shown in Figure 1d) with 6 Gy of IR and evaluated apoptosis 6 h later. Consistent with the role of *Wip1* in regulation of the DNA damage response, inactivation of only *Chk2* and *p53* reduced apoptosis in *Wip1/Chk2* and *Wip1/p53* double-deficient mice, respectively (Figure 2d). p38, *Cdkn2a*, and *Gadd45a* did not seem to execute the effects of IR on ISC apoptosis.

Tumors in *Apc<sup>Min</sup>* mice arise from homozygous loss of the wild-type *Apc<sup>Min</sup>* allele, resulting in constitutive activation of the  $\beta$ -catenin-Tcf/Lef signaling pathway.<sup>26</sup> This activation may trigger an efficient p53 response, as does IR.<sup>17,26</sup> To investigate how activation of  $\beta$ -catenin signaling modulates intestinal apoptosis, we treated wild-type and *Wip1*-deficient mice with Gsk3 inhibitor IX. We previously found that this inhibitor upregulates Tcf/Lef-dependent genes such as *Ccnd1* (cyclin D1), *Myc*, and *Cd44*, and induces ISC apoptosis in *Wip1*-deficient mice.<sup>6</sup> However, inhibitor treatment was not sufficient to activate ISC apoptosis in wild-type mice.<sup>6</sup> Next, we turned to the analysis of *Wip1*-deficient mice crossed into various genetic backgrounds (Figure 2e). *Wip1/Cdkn2a* and *Wip1/Gadd45a* double-mutant mice did not activate apoptosis after treatment with the inhibitor of Gsk3. Deletion of *Chk2* and *p53* reduced basal as well as Gsk3 inhibitor IX-induced apoptosis in *Wip1*-deficient mice, while inactivation of *p38* had no effect. Taken together, our observations of the effects of

chemical treatment and various genetic manipulations revealed that *Wip1* deficiency activates basal apoptosis of ISCs in an ATM-*Chk2*-p53-dependent manner, while Gsk3 inhibitor IX-induced apoptosis is regulated through *Cdkn2a* and *Gadd45a*.

***Cdkn2a*, *Chk2*, and *Gadd45a* are involved in *Wip1*-dependent regulation of *Apc<sup>Min</sup>*-driven polyposis.** We previously found that the ability of ISCs to undergo apoptosis in *Wip1*-deficient mice is an important initial step in suppression of ISC conversion into tumor-initiating cells and subsequent polyp formation. To elucidate the roles of various signaling pathways in the regulation of *Wip1*-dependent suppression of polyposis, various genetically modified mice were backcrossed into a *Wip1/Apc<sup>Min</sup>* background, and polyp formation was assessed in these mice at day 90. Deletion of *Cdkn2a*, *Chk2*, and *Gadd45a* reversed the tumor-resistant phenotype of *Wip1/Apc<sup>Min</sup>* mice (Figure 3a). The average numbers of polyps in *Wip1/Cdkn2a/Apc<sup>Min</sup>*, *Wip1/Chk2/Apc<sup>Min</sup>*, and *Wip1/Gadd45a/Apc<sup>Min</sup>* mice were 49, 43, and 68, respectively. On the other hand, inactivation of p38Mapk did not affect polyposis, as *Wip1/p38<sup>Kl/+</sup>/Apc<sup>Min</sup>* and *Wip1/Apc<sup>Min</sup>* mice had similar, low numbers of lesions (Figure 3a).

Deletion of *Cdkn2a*, *Chk2*, and *p53* efficiently abrogates the tumor-resistant phenotype of *Wip1*-deficient mice in the model of *Apc<sup>Min</sup>*-induced polyposis; these observations are not unexpected, as disruption of these molecules attenuates



**Figure 3** Multiple signaling pathways contribute to suppression of polyposis in *Wip1*-deficient mice. Mice of different genetic backgrounds, as shown in the panels a and b, were analyzed for polyp formation at day 90. Significance (relative to *Wip1*-deficient mice) levels: \*\*\* $P < 0.001$



activation of p53. On the other hand, we were intrigued by our observation that *Gadd45a* deletion reverses the tumor-resistant phenotype of *Wip1*-deficient mice, and we asked whether the presence of a single allele impacted tumorigenesis. Deletion of a single *Gadd45a* allele also reversed the tumor-resistant phenotype in *Wip1*-deficient mice (Figure 3b); the number of polyps that developed in *Wip1/Apc<sup>Min</sup>* mice heterozygous for *Gadd45a* was similar to the numbers in *Apc<sup>Min</sup>* and *Gadd45a/Wip1/Apc<sup>Min</sup>*-deficient mice, indicating that *Gadd45a* is haploinsufficient in the *Wip1*-dependent regulation of *Apc<sup>Min</sup>*-induced polyposis.

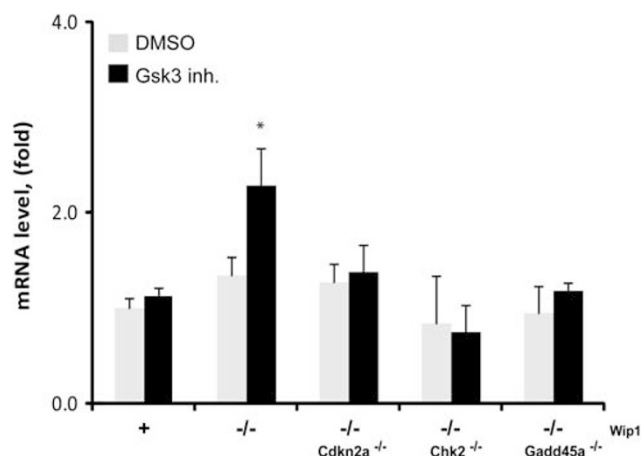
**p53 activation decreases following  $\beta$ -catenin activation in mice with *Cdkn2a*, *Chk2*, or *Gadd45a* deletion.** p53 regulates the conversion of stem cells into tumor-initiating cells, protecting against polyp formation in *Apc<sup>Min</sup>* mice.<sup>6</sup> *Cdkn2a* (through *Arf*) and *Chk2* kinase are known positive regulators of p53 that mediate efficient p53 activation in the presence of various oncogenes.<sup>27,28</sup> We checked whether disruption of *Cdkn2a*, *Chk2*, or *Gadd45a* resulted in attenuation of p53 activity in control mice and after treatment with Gsk3 inhibitor IX. We previously showed that induction of a p53 transcriptional target, p21, is fully dependent on the presence of p53 in the mouse intestine and may serve as a read-out for p53 activity, which is otherwise difficult to evaluate *in vivo*.<sup>6</sup> p21 mRNA levels in *Cdkn2a/Wip1/Apc<sup>Min</sup>* and *Chk2/Wip1/Apc<sup>Min</sup>*-deficient mice were attenuated when compared to *Wip1/Apc<sup>Min</sup>* mice (Figure 4). In a similar manner, we observed lack of activation of p21 mRNA in *Gadd45/Wip1/Apc<sup>Min</sup>* mice, suggesting that the presence of *Gadd45a* is required for efficient p53 activation in response to stabilization of  $\beta$ -catenin. Thus, *Chk2*, *Cdkn2a*, and *Gadd45a* are required for efficient activation of p53 in the intestine of *Wip1*-deficient mice after treatment with an inhibitor of Gsk3.

***Gadd45a* is required for Jnk activation.** Although *Gadd45a* has been previously implicated in the positive regulation of

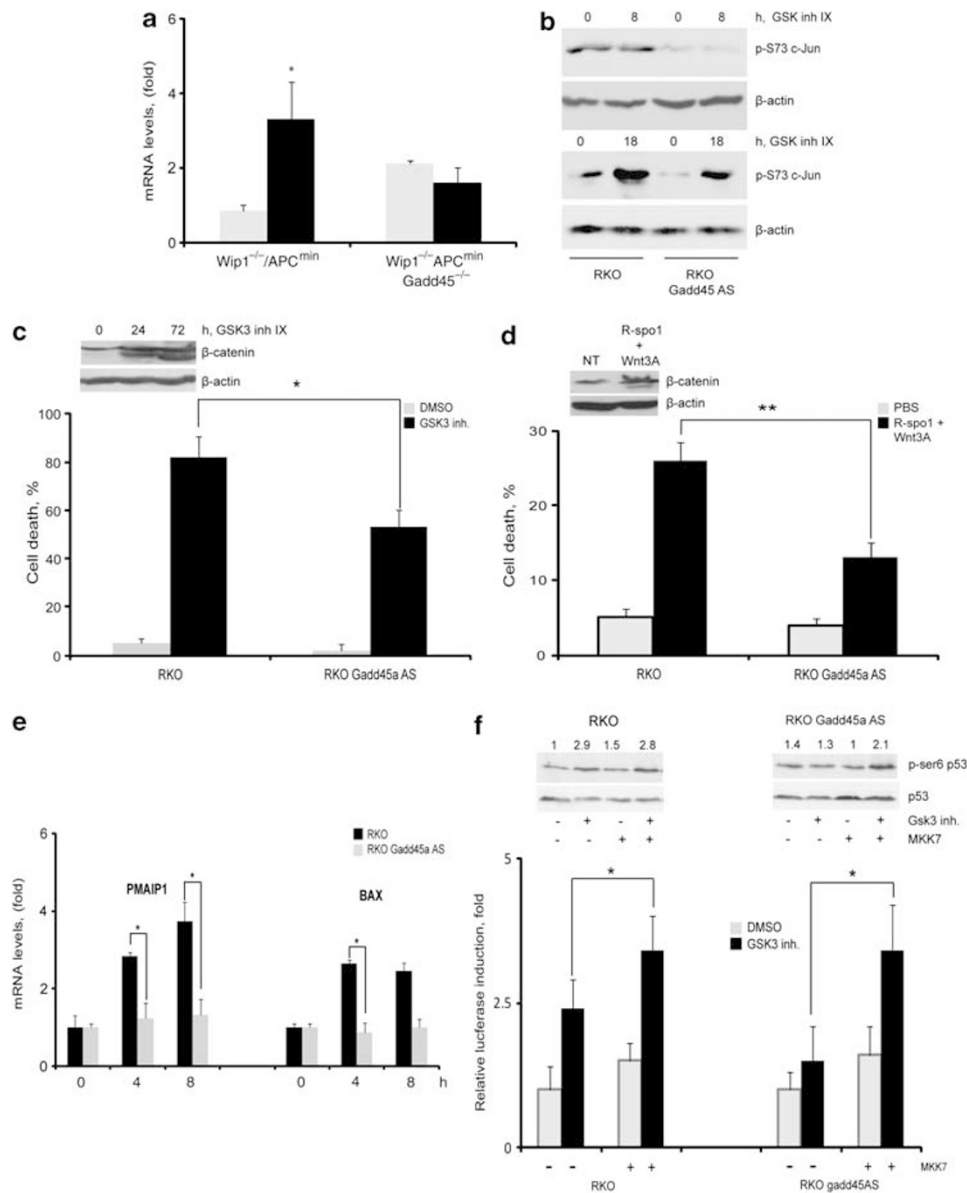
p53, it is unknown how *Gadd45a* deficiency attenuates ISC apoptosis and reduces p53 activation in the presence of active  $\beta$ -catenin. *Gadd45a* has been previously implicated in the regulation of the stress-induced kinases Jnk and p38Mapk.<sup>19–21</sup> As we determined that p38MAPK has no role in regulation of ISC apoptosis and intestinal tumorigenesis in *Wip1*-deficient mice (Figures 2 and 3), we concentrated on the Jnk-dependent pathway. Activation of the Jnk pathway has been implicated in p53 activation by various stresses; as reported previously, Jnk may mediate *Gadd45a* proapoptotic functions in some cell types,<sup>20,29</sup> and thus be responsible for enhanced p53 activation after treatment with Gsk3 inhibitor IX in *Wip1*-deficient mice. To further evaluate this hypothesis, we analyzed the Jnk pathway in the intestinal epithelia of mice treated with Gsk3 inhibitor IX. Active Jnk activates c-Jun, a part of the AP1 transcriptional complex, and in turn c-Jun is under the control of AP1. We found that the expression of c-Jun mRNA increased when the mice were injected with the inhibitor, an upregulation that was fully dependent on the presence of *Gadd45a*, as its deletion efficiently reduced c-Jun mRNA upregulation in the intestine (Figure 5a).

To illustrate the relevance of our findings to human cancer, we analyzed the human RKO colorectal cell line. We used a subclone of RKO cells with a stably integrated *Gadd45a* antisense molecule to lower *Gadd45a* expression. Both cell lines were treated with Gsk3 inhibitor IX and evaluated for c-Jun phosphorylation as a read-out of Jnk-dependent signaling. We also assayed for activation of apoptosis. The basal level of c-Jun phosphorylation was significantly reduced in RKO *Gadd45a* antisense cells (Figure 5b). Further, induction of c-Jun phosphorylation 18 h after treatment with Gsk3 inhibitor IX was also significantly lower in RKO *Gadd45a* antisense cells *versus* the control cell line. Gsk3 inhibitor IX treatment induced efficient apoptosis of RKO cells, which was significantly attenuated when *Gadd45a* expression was silenced by the antisense (Figure 5c). In a similar manner, we found that treatment of RKO cells with R-spondin and Wnt3a ligand results in efficient accumulation of  $\beta$ -catenin and this was sufficient to activate a robust apoptosis in RKO cells, but not in cells expressing *Gadd45a* antisense (Figure 5d). The attenuation of cell death by *Gadd45a* antisense was also accompanied by loss of induction of p53 target proapoptotic genes *Pmaip1* and *Bax* after Gsk3 inhibition (Figure 5e).

To understand whether lack of Jnk signaling is responsible for reduced activation of p53 in *Gadd45a* AS RKO cells, next we transfected cells with a vector carrying an upstream activator of Jnk, mitogen-activated protein kinase kinase 7 (MKK7), and analyzed activation of p53 under different conditions. We found that both p53 phosphorylation at activating site, Ser6,<sup>30</sup> and p53 reporter activity were robustly induced in the presence of Gsk3 inhibitor only in RKO but not in RKO *Gadd45a* AS cells (Figure 5f). In turn, introduction of MKK7 was able to enhance both p53 Ser6 phosphorylation and reporter activity in RKO *Gadd45a* AS cells to the level comparable with RKO cells (Figure 5f). Our data are consistent with the recent report regarding the role of MKK7 in regulation of p53 activation and tumorigenesis.<sup>30</sup> Thus, attenuation of *Gadd45a* levels leads to decreased activation of the Jnk—p53 pathway and lowers the level of apoptosis in



**Figure 4** p53 activation in *Wip1*-deficient mice after the treatment with Gsk3 inhibitor IX is dependent on *Cdkn2a*, *Chk2*, and *Gadd45a*. Induction of p21 mRNA as a read-out of p53 activity *in vivo* was analyzed by real-time RT-PCR in the intestinal crypts 8 h after the treatment with Gsk3 inhibitor IX. Significance (relative to wild-type mice treated with Gsk3 inhibitor) level: \* $P < 0.05$



**Figure 5** Gadd45a regulates Jnk signaling pathway. (a) The level of expression of c-Jun mRNA as a read-out of jnk activity *in vivo* was analyzed by real-time RT-PCR in intestinal crypts of different genotypes after the treatment with Gsk3 inhibitor IX (black columns). Statistical significance was analyzed relative to the level of c-jun in non-treated Wip1-deficient mice (white columns). (b) Phosphorylation of Jnk target c-Jun at serine 73 was analyzed in RKO and RKO Gadd45a AS cells after the treatment with Gsk3 inhibitor IX after 8 h (upper panel) and 18 h (lower panel). (c) Accumulation of  $\beta$ -catenin was analyzed by western blotting after the treatment of RKO cells with GSK inhibitor IX (top panel). Cell death was analyzed by GuavaViaCount as described in Materials and Methods in RKO and RKO Gadd45a AS cells 18 h after the treatment with Gsk3 inhibitor IX (lower panel). Significance level:  $*P < 0.05$ . (d) Accumulation of  $\beta$ -catenin was analyzed by western blotting after the treatment of RKO cells with R-spondin and Wnt3a ligand (top panel). Cell death was analyzed by GuavaViaCount in RKO and RKO Gadd45a AS cells 18 h after the treatment with R-spondin and Wnt3a ligand (bottom graph). Significance level:  $**P < 0.01$ . (e) Attenuated induction of p53 targets genes *PMAIP1* and *BAX* at 4 and 8 h after the treatment with Gsk3 inhibitor IX in RKO cells with Gadd45a antisense. mRNA levels were analyzed by real-time RT-PCR. Significance level:  $*P < 0.05$ . (f) Analysis of p53 phosphorylation at Ser6 was carried out in RKO and RKO Gadd45a AS cells that were either treated with Gsk3 inhibitor XI, transfected with MKK7 vector or both. In parallel, the level of p53 transactivation activity was analyzed under different conditions using Luciferase reporter containing p53 response elements in the promoter as described in the panel and Materials and Methods. Significance level:  $*P < 0.05$

response to treatment with Gsk3 inhibitor IX, both in mouse (Figures 2-4) and in human (Figure 5) cells.

## Discussion

Recent studies have revealed the existence of tumorigenesis barriers that slow or inhibit the progression of preneoplastic lesions to neoplasia.<sup>27,31</sup> Activation of a DNA damage

response represents one of such barriers that is operational in various cancers, including colorectal tumors.<sup>27</sup> Inhibition of the DNA double-strand break response kinase ATM in a mouse model led to increased tumor size and invasiveness.<sup>32</sup> Modulating the conditions of enhanced DNA damage responses may therefore provide an interesting approach for understanding tumor evolution as well as the sequence of events that must occur for tumor progression.

p53 is a major tumor suppressor that is frequently mutated in human colorectal cancers; however, this genetic change occurs only in later stages of tumorigenesis.<sup>33</sup> Consistent with p53 function only in advanced colorectal cancer, p53 deletion does not affect the number of polyps in the *Apc<sup>Min</sup>* mouse model of intestinal tumorigenesis,<sup>6</sup> which mimics pathogenesis of familial adenomatous polyposis in humans. In contrast, emerging data strongly support the hypothesis that p53 upregulation may efficiently suppress intestinal tumorigenesis. Deletion of *Mdm2* or *Wip1* phosphatase, two negative regulators of p53, results in p53 upregulation and suppression of *Apc<sup>Min</sup>*-induced polyposis in mice.<sup>6,34</sup> This effect, at least in *Wip1*-deficient mice, appeared to be at the level of regulation of ISC conversion into cancer stem cells.<sup>6</sup>

It has become increasingly clear that a primary function of *Wip1* is the repression of several signaling pathways within the DNA damage-response network in which various molecules have been shown to be direct targets of *Wip1*. In addition to inhibiting p38MAPK after UV radiation,<sup>23</sup> *Wip1* has been shown to dephosphorylate numerous molecules within the ATM-dependent signaling network including ATM itself<sup>8,16</sup> and *Chk2*,<sup>9</sup> downstream of ATM. On the basis of these observations, we used *Wip1*-deficient mice as a model system to understand the contribution of an enhanced DNA damage response to the initial steps of tumorigenesis.

If DNA damage occurs during the early stages of colorectal cancer, the tumor cell must find ways to overcome this barrier in order to develop into a full-blown cancer. In many instances, this initial step involves the loss or mutation of p53, which appears not to be the case for human colorectal cancer.<sup>33</sup> Here we identified loss of *Gadd45a* to be essential for cell to survive p53 activation in response to active  $\beta$ -catenin signaling pathway. We demonstrated that *Gadd45a* feeds into the Jnk-dependent signaling pathway to activate p53 when  $\beta$ -catenin is active. Consistent with this observation, *Gadd45a* deletion accelerates Ras- and Myc-driven mammary tumor formation, potentially through regulation of Jnk-induced apoptosis,<sup>29,35</sup> while MKK7–Jnk signaling pathway is required for activation of p53 in several mouse models of cancer.<sup>30</sup>

It is important to note that *Gadd45a* deletion specifically affected  $\beta$ -catenin-induced activation but did not completely inactivate the Jnk-dependent signaling pathway. As a result, activation of ISC apoptosis induced by Gsk3 inhibitor IX was specifically diminished in *Gadd45a*-deficient cells. In contrast, full inactivation of Jnk may exert the opposite effect and suppress *Apc<sup>Min</sup>*-induced tumorigenesis through inactivation of TCF4-dependent transcription.<sup>36</sup> In the case of *Gadd45a*-deficient mice, Gsk3 inhibitor-induced Jnk activation was reduced; however, activation of TCF4 downstream targets such as c-Myc, cyclin D1, and CD44 was comparable with other genotypes (data not shown). Thus, lack of *Gadd45a* may provide a selective advantage for ISC survival and tumor development through suppression of Jnk-dependent activation of p53 when  $\beta$ -catenin is stabilized.

In conclusion, our extensive genetic analysis of *Apc<sup>Min</sup>* mice under conditions of an enhanced DNA damage response in *Wip1*-deficient mice underlines the importance of *Gadd45a* in the suppression of intestinal tumorigenesis. Loss of *Gadd45a* may therefore be an early event in the course of human colorectal cancer.

## Materials and Methods

**Mouse strains.** All animal protocols used in this study were approved by the Institute of Molecular and Cell Biology Animal Safety and Use Committee. *Wip1*<sup>-/-</sup>, *p53*<sup>-/-</sup>, *ATM*<sup>-/-</sup>, *Chk2*<sup>-/-</sup>, *Cdkn2a*<sup>-/-</sup>, and p38 knock-in mice were previously described.<sup>6,8,25,29</sup> *Apc<sup>Min</sup>* heterozygous and Lgr5-EGFP-IRES-creERT2 mice<sup>13</sup> were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). We crossed *Wip1*<sup>+/-</sup> mice with *Apc<sup>Min</sup>* with mice of different genotypes to obtain mice for the analysis in this study. Mice were euthanized and polyps were counted at day 90.

**Immunohistochemistry.** Formalin-fixed paraffin-embedded mouse intestinal samples were sectioned at 5  $\mu$ m and mounted on coated slides. Following dewaxing and re-hydration, sections were pretreated with peroxidase blocking buffer (3% H<sub>2</sub>O<sub>2</sub> in methanol) for 20 min at room temperature. Antigen retrieval was performed by autoclaving samples in Sodium Citrate buffer (10 mM, pH 6.0) for 30 min. Blocking of nonspecific binding was performed using 3% BSA or 5% blocking reagent (Roche, Indianapolis, IN, USA) for 30 min at RT. The rabbit anti-caspase-3 (1:300; AF835, R&D Systems, Minneapolis, MN, USA) were used in all experiments. The Envision + kit (Dako, Glostrup, Denmark) was used as a secondary reagent.

To analyze apoptosis in Lgr5-EGFP-IRES-creERT2 mice, small intestine was cut into 12 segments with equal length, aligned parallel and cryo-sectioned to 18- $\mu$ m coronal sections. TUNEL assay was performed according to the manufacturer protocol (Millipore, Billerica, MA, USA, S7165). Briefly, sections were washed in PBS three times then immersed in equilibration buffer for 1 min. After removal of the equilibration buffer, terminal deoxynucleotidyl transferase enzyme in reaction buffer was applied to label damaged DNA with dUTP-digoxigenin. Reaction was carried out in 37 °C for 2 h, and an anti-digoxigenin antibody conjugated with a rhodamine fluorochrome was used to visualize apoptotic cells. Cell number and positions and co-localization with EGFP was analyzed using the inverted confocal Zeiss (Berlin, Germany) LSM700 and LSM image browser.

**Real time RT-PCR.** mRNA expression was determined by Real-Time PCR. Tissues were frozen in dry ice and RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using Superscript II (Invitrogen) according to the manufacturer instructions. Real-Time PCR was performed at LightCycler 2.0 Real-Time PCR System (Roche) with LightCycler FastStart DNA MasterPlus SYBR Green I (Roche). *GAPDH* and *HRPT* mRNA levels were used as a reference. We used the following primers: p21 5'-TTGCACTCTGGTGTCTGAGC-3'; 5'-AGGGCCCTACCGTCTACTA-3'. c-jun, 5'-AGCAGGGACCCATGGAAGTT-3', and 5'-AAAGATGACCTTTGCTTGCA T-3'. To analyze *PMAIP1*, *BAX*, Actin mRNA expression in RKO cells, we used Solaris Gene Expression Assay (Dharmacon, Lafayette, CO, USA) and 7500 Fast Real-time PCR system (Applied Biosystem, Foster City, CA, USA).

**Inhibitors.** Mice were injected i.p. with the Rapamycin<sup>37</sup> (Sigma-Aldrich, St. Louis, MO, USA, #R0395) 7.5 mg per kg of body weight in 5% DMSO or AKT inhibitor<sup>38</sup> (NSC 154020, Calbiochem, Millipore) 20 mg per kg of body weight in 5% DMSO or ATM inhibitor (KU55933, Calbiochem, Millipore) 15 mg per kg of body weight in 5% DMSO or DNA-PK inhibitor<sup>39</sup> (NU7026, Sigma-Aldrich) 15 mg per kg of body weight in 5% DMSO or p38MAPK inhibitor<sup>12</sup> (SB202190, Sigma-Aldrich) 10 mg per kg of body weight in 5% DMSO, or GSK-3 inhibitors IX<sup>6</sup> (Calbiochem, Millipore) 15 mg per kg of body weight in 5% DMSO. After 6, 10, or 12 h, mice were euthanized and organs were collected for subsequent analysis. To induce activation of  $\beta$ -catenin RKO and RKO Gadd45 AS cells were treated with 10- $\mu$ M GSK-3 inhibitors IX (Calbiochem, Millipore) or with combination of 500 ng/ml R-spondin1 and 200 ng/ml Wnt3A (both from R&D Systems).

**Cell death assay.** The number of dead cells was estimated by the cell death assay using the Guava ViaCount Reagent (Millipore). Cells were trypsinized, washed with PBS solution, and stained with Guava ViaCount Reagent for 5 min. The percentage of dead cells was measured by using Guava EasyCyte Plus Flow Cytometry System (Millipore) and analyzed by using Guava ViaCount Software.

**Luciferase assay.** Transient transfections of RKO and RKO Gadd45 AS cells were performed using JetPEI (Polyplus, Illkirch, France), 2000 ng of pBabe or pBabe-MKK7, 400 ng of the p53-3xRE-luc Firefly luciferase reporter construct and 20 ng pRL-TK Renilla luciferase as reference vector (Promega, Madison, WI, USA) per 5  $\times$  10<sup>5</sup> cells. Thirty hours after transfection, cells were treated with GSK3



inhibitor IX (Calbiochem, Millipore). After additional 16 h of incubation, cells were collected and measured for Firefly and Renilla luciferase activities using the Dual Luciferase Assay System (Promega) on LUMAT LB9507 luminometer. Results represent the average standard deviations of two independent experiments.

**Western blot analysis.** The RKO and RKO gadd45 AS cell lines were purchased from ATCC (Manassas, VA, USA). Cell lysates containing 50  $\mu$ g of protein were analyzed by western blot using anti-phospho-c-Jun (Ser73) antibody, anti-phospho-p53 (Ser6) antibody (Cell Signaling, Danvers, MA, USA), anti-p53 DO1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti- $\mu$ -catenin (BD Transduction Laboratories, San Jose, CA, USA) and anti-actin antibody (Sigma-Aldrich, A 2103). To analyze phosphorylation status of p53 in human cells, RKO cells were treated with 25  $\mu$ M of proteasomal inhibitor MG-132 (Sigma-Aldrich) for 3 h before harvesting.

Small intestinal crypts were isolated from adult mice of 6–12 weeks as described before.<sup>8</sup> Crypt lysates containing 100  $\mu$ g of protein were analyzed by western blot using anti-phospho-p53 (Ser18) (AnaSpec, Fremont, CA, USA) and anti-p53 CM5 (Novocastra, Newcastle upon Tyne, UK). Secondary antibodies were from Jackson Immuno Research (West Grove, PA, USA). Immunoreactivity was detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology). We used GelQuant.NET software provided by biochemlabsolutions.com to calculate relative intensity of bands. p-Ser6 p53 band average-corrected intensity by total p53 band was presented.

### Conflict of Interest

The authors declare no conflict of interest.

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